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Report

Absence of human papillomavirus DNA in breast cancer as revealed by polymerase chain reaction

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Summary

Oncogenic human papillomavirus (HPV) types 16 and 18 commonly associated with cervical cancer are found in many epithelial malignancies at extra-genital sites including breast. The transforming gene products of HPV have also been shown to immortalize breast epithelial cells *in vitro*. But the findings of HPV DNA in breast carcinoma are found to be contradictory. In the present study fine needle aspirate cell (FNAC) samples from 26 breast cancer patients and four breast tumour biopsies were analysed for the presence of HPV 16 and 18 DNA sequences by both polymerase chain reaction (PCR) and Southern blot hybridization. Of 26 fine needle aspirate cell samples and four breast cancer biopsies, not a single sample was found to be positive by either PCR or Southern blot hybridization. The observation of complete absence of HPV DNA sequences in breast cancer refute the possibility of any role for oncogenic genital HPV types 16 and 18 in the pathogenesis of breast cancer.

Introduction

More than 75 human papillomavirus (HPV) DNA types thus far described have been associated with a variety of epithelial cancers at different organ sites in humans [1–5]. Of about 20 HPVs that have been associated with lesions of the anogenital tract, HPV types 16 and 18 are considered to be 'high risk' types and are strongly implicated in the development of anogenital cancers [6–9]. Furthermore, the oncoproteins E6 and E7 from these two viruses have been shown to immortalise human genital keratinocytes *in vitro* [10–12]. Recently it has been demonstrated that the HPV 16 and 18 genes can also immortalise breast epithelial cells [13, 14].

Since these genital HPVs are detected in tumours at distant organ sites such as larynx, oesophagus and anus [2–4, 15, 16], there have been attempts to demonstrate HPV DNA sequences in breast carcinomas. While Di-Lonardo *et al.* [17] using PCR detected HPV 16 DNA in 29.4% of cases, Wrede *et al.* [18] failed to demonstrate either HPV 16 or 18 DNA in a series of breast cancer specimens.

Because of these conflicting results and since prevalence of HPV, particularly the type 16, is exclusively high in Indian women with anogenital cancers [19], we have used both polymerase chain reaction (PCR) and Southern blot hybridization to investigate fine needle aspirate cell (FNAC) samples and biopsies from breast cancer patients for the presence of HPV 16 and 18 DNA sequences.

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Materials and methods

Fine needle aspirate cell (FNAC) samples from 26 breast cancer patients and four breast cancer biopsies were employed for analysis of HPV 16 and 18 DNA sequences. Samples were collected from women who were diagnosed to have breast carcinoma during this study period of 7 months in aspiration clinics of our Institute (ICPO), University College of Medical Sciences, and Lok Nayak Jaiprakash Narayan Hospital, New Delhi. The clinical details of the patients are presented in Table 1. An aliquot of each FNAC sample was used for cytological slide preparation for cytodiagnosis and the rest was collected in an eppendorf tube containing phosphate buffered saline (pH 7). Care was taken to wash the needle to ensure collection of left-over aspirated cells attached along the walls of the needle and syringe. FNAC samples were stored at – 70° C until analysis.

DNA extraction

DNA extraction was done by non-organic methods [20] to ensure high recovery of DNA. Briefly, the methods involved washing of cell pellets twice in cold PBS solution and twice in 1 ml Tris-Triton buffer (TTB) containing 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 300 mM sucrose, and 0.8% Triton X100. The pellet collected by microfuging was rewashed in 500 µl cold TE buffer (TEB) containing

Table 1. Clinical, cytological, and molecular virological parameters of the patients of breast carcinoma

Patient no.	Age (yrs)	Menopausal status	Clinical diagnosis	Cytological diagnosis	HPV 16 DNA by PCR
1.	36	Pre	Benign	Fibroadenoma	-ve
2.	50	Post	Malignant	Infilt. duct Ca	-ve
3.	35	Pre	Benign	Fibroadenoma	-ve
4.	35	Pre	Benign	Fibroadenoma	-ve
5.	35	Pre	Malignant	Infilt. duct Ca	-ve
6.	48	Post	Malignant	Infilt. duct Ca	-ve
7.	42	Pre	Benign	Infilt. duct Ca	-ve
8.	60	Post	Benign	Infilt. duct Ca	-ve
9.	60	Post	Malignant	Infilt. duct Ca	-ve
10.	40	Pre	Malignant	Infilt. duct Ca	-ve
11.	45	Pre	Malignant	Infilt. duct Ca	-ve
12.	50	Post	Malignant	Mucinous Ca	-ve
13.	45	Pre	Malignant	Infilt. duct Ca	-ve
14.	40	Pre	Malignant	Infilt. duct Ca	-ve
15.	44	Pre	Malignant	Fibroadenoma	-ve
16.	60	Post	Malignant	Infilt. duct Ca	-ve
17.	34	Pre	Malignant	Recurrent duct Ca	-ve
18.	45	Pre	Malignant	Recurrent duct Ca	-ve
19.	36	Pre	Malignant	Infilt. duct Ca	-ve
20.	55	Post	Malignant	Infilt. duct Ca	-ve
21.	40	Pre	Malignant	Infilt. duct Ca	-ve
22.	45	Pre	Malignant	Infilt. duct Ca	-ve
23.	50	Post	Malignant	Infilt. duct Ca	-ve
24.	48	Post	Malignant	Infilt. duct Ca	-ve
25.	44	Pre	Malignant	Infilt. duct Ca	-ve
26.	42	Pre	Malignant	Infilt. duct Ca	-ve
27.	35	Pre	Malignant	Infilt. duct Ca	-ve
28.	40	Pre	Malignant	Infilt. duct Ca	-ve
29.	45	Post	Malignant	Infilt. duct Ca	-ve
30.	48	Post	Malignant	Infilt. duct Ca	-ve

10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM NaCl. Finally, the pellet was resuspended in 200 µl of TE buffer supplemented with 1.25 mg/ml. Proteinase K (Boehringer Mannheim, Germany) and incubated at 65° C in a water bath for 2 to 3 hrs till we get a transparent solution. This DNA was directly used for PCR amplification following measurement of DNA concentration in an ethidium bromide-stained minigel.

Polymerase chain reaction

For PCR, about 1 µg of breast cancer DNA samples was amplified along with β-globin gene as an experimental internal control. HPV 16 positive cervical tumour DNA and human placental DNA were used as positive and negative controls for HPV respectively. The details of oligonucleotide primers used for amplification of HPV 16, 18, and β-globin gene are presented in Table 2. The amplification methods were as described by Saiki *et al.* [21, 22] with certain modifications [23, 24]. The reaction was carried out in 50 µl l total reaction mix containing, 1 µg breast carcinoma DNA, 67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.7 mM ammonium sulphate, 10 mM β-mercaptoethanol, 6.7 mM EDTA (pH 8.0), 0.17 µg/ml BSA, 2.5 mM dNTP (dATP, dCTP, dGTP, dTTP), 1 µM HPV primers, 1 µM β-globin primers, 3 µl DMSO, and 1 µl (5 units/µl) of Taq DNA polymerase (Cetus Corporation, USA). The reaction mix was overlayed with 50 µl mineral oil (Cetus) to prevent evaporation. PCR was done using a DNA Thermal Cycler of Perkin Elmer Cetus, USA. Tumour DNA was first denatured at 94° C for 5 min, annealed at 55° C for 1 min, followed by extension for 2 min at 72° C. These steps were repeated for an additional 35 cycles with denaturation (94 ° C) for 1 min only. In the last cycle, the extension was allowed for 7 min. 20 µl of amplified products were run on 3% Nusieve-agarose gel, stained in ethidium bromide, and photographed by a UV transilluminator.

HPV 16 and 18 primers were used separately. HPV 16 positive cervical tumour DNA or HPV 16 plasmid DNA and HPV 18 positive HeLa DNA were used as positive controls for HPV 16 and 18 respectively.

Southern blotting and hybridization

5–10 micrograms of tumour DNA was cleaved with Bam H1 (a single-cut restriction enzyme for HPV 16, Boehringer Mannheim, Germany) according to manufacturer's protocol. Digested DNA was separated by 1% agarose gel electrophoresis and blotted onto Gene Screen Plus™ membrane (NEN Research Products, Du Pont, Boston, USA) as described by Southern [25] with some modifications [19]. Vector-free HPV 16 and 18 inserts were random-primed with ^{32}P dCTP (specific activity, 3000–4000 Ci mmol, Bhabha Atomic Research Centre, Bombay, India) to specific activity more than 10^8 dpm/µg of DNA. Blots were hybridized under non-stringent conditions (Tm-40° C) at 42° C (50% formamide). HPV 16/18 and all related HPV DNA sequences. Following washings (48° C), the filters were air dried and exposed to Kodak X-OMAT-AR X-ray film with intensifying screen at – 70° C.

Table 2. Specification of oligonucleotide primers for polymerase chain reaction

Primer code	Genome region	Nucleotide sequence	Nucleotide sequence localization	Amplimer (bp)
HPV 16 P1	URR	5'-AAGGCCAACTAAATGTCAC-3'	7763-7781	217
HPV 16 P2	URR	5'-CTGCTTTTATACTAACCGG-3'	57-75	
HPV 18 P1	E6	5'-ACCTTAATGAAAAACCACGA-3'	463-482	100
HPV 18 P2	E6	5'-CGTCGTTTAGAGTCGTTCCTG-3'	543-562	
β-globin 1		5'-GAAGAGCCAAGGACAGGTAC-3'		268
β-globin 2		5'-CAACTTCATCCACGTTACACC-3'		

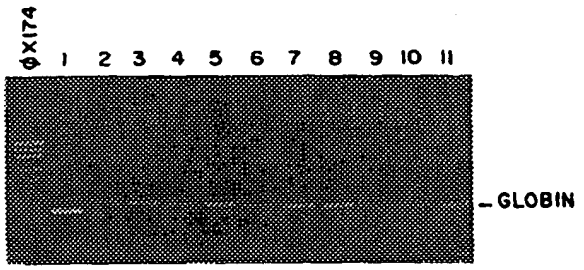

Fig. 1. PCR amplification of HPV 16 DNA and β-globin gene sequences in breast cancer biopsy DNA. Lanes 2–11 are breast cancer FNAC DNA samples showing amplification of only β-globin gene (268 bp) and no HPV DNA sequences. Lane 1 is a cervical cancer sample showing amplification of both HPV 16 (217 bp) and beta globin gene sequences. At the extreme left, Hae III-digested ΦX 174 DNA molecular weight marker.

Results

Since the specific oligonucleotide primer-directed amplification in polymerase chain reaction (PCR) is the most sensitive technique, thirty primary breast tumour DNAs including four breast cancer biopsy DNAs were first analysed by PCR for the presence of HPV DNA sequences. Two pairs of oligonucleotide primers – one from the most conserved upstream regulatory region (URR) and the other from the transforming E6 region of HPV 16 and 18 (see Table 2), were used for the detection of HPV DNA sequences. A pair of primers that amplify a 268 bp fragment of normal human β-globin gene was used as an experimental control. HPV 16 positive cervical tumour DNA and human placental DNA served as the positive and negative controls respectively. No breast carcinoma DNA sample was found to be positive for either HPV 16 or HPV 18 DNA sequences. But the results of positive and negative controls were obtained as expected. In Fig. 1, HPV 16 positive cervical tumour DNA showed amplification of both 217 bp HPV 16 as well as 268 bp β-globin gene sequences in lane 1 while in lane 2, only a single 268 bp amplification of the β-globin gene was seen for placental DNA. Similarly, all breast cancer DNA showed clear amplification of the control β-globin gene of the expected size of 268 bp only (see Table 2, Fig. 1). This indicated that the quality and quantity of tumour DNA was sufficient for the amplification reaction but no HPV DNA was present.

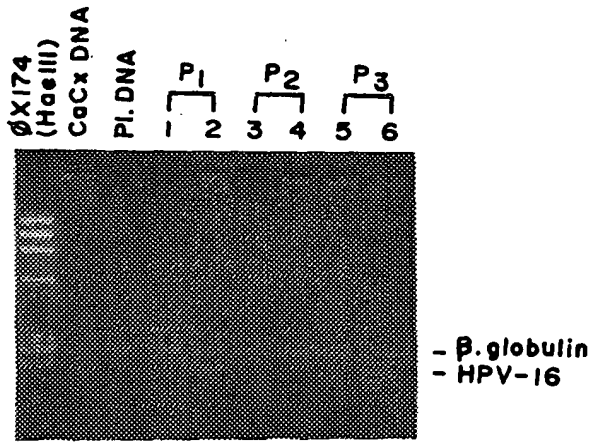

Fig. 2. PCR amplification of HPV 16 DNA along with β-globin gene sequences in FNAC and cervical scrapes of the same patient. First lane is Hae III-digested ΦX 174 DNA size marker. Next two lanes are cervical cancer DNA and placental DNA showing amplification of both HPV 16 (217 bp) and β-globin on only β-globin gene, respectively. The lanes 1, 3, and 5 are FNAC DNA samples in patients 1, 2, and 3 respectively showing only β-globin gene but no HPV DNA sequences. The lanes 2 and 6 are patients 1 and 3, scraped cervical cell DNA samples showing amplification of both β-globin and HPV 16 DNA sequences, while lane 4 shows only β-globin amplification. P1, P2, P3 = Number of patients.

To ensure further, gel-separated PCR products as well as breast cancer DNA samples were hybridized by ^{32}P-labelled 8 kb HPV 16 and HPV 18 DNA inserts under both stringent (Tm-20° C and 50% formamide) and non-stringent (Tm-40° C and 20% formamide) conditions to detect specific and related or yet uncharacterised HPV types. But no tumour DNA sample was found to harbour any HPV type (Figs not shown). These observations were indicative of the fact that no HPV type is involved during carcinogenesis of human breast. Furthermore and most importantly, two (33%) of six breast carcinoma patients whose cervical scrapes were available for HPV detection, showed presence of HPV 16 DNA sequences (see Fig. 2). Therefore, HPV may even be present in cervical epithelium but not in the breast of the same woman.

Discussion

Since the association of genital human papillomavirus with extragenital sites and their involvement in immortalisation of breast epithelial cells have been demonstrated, Di-Lonardo *et al.* [17] used PCR to detect HPV DNA sequences in breast carcinoma biopsy specimens. They reported presence of only HPV 16 DNA sequences in 29.4% of breast carcinoma. The present study, however, shows that the high risk HPV types 16 and 18 which are most commonly associated with anogenital cancers, are absent in fine needle aspirate samples as well as biopsies of breast carcinomas of Indian women. Our results are in good agreement with those of Wrede *et al.* [18], who also failed to detect HPV DNA sequences in a series of breast carcinoma biopsies. Our results are further confirmed by Southern blot hybridization, which also failed to detect HPV 16 or 18 or related HPV DNA sequences in breast cancer samples. Similar results were obtained by Ostrow *et al.* [26] for primary breast cancer specimens. It is interesting to note that although Di-Lonardo *et al.* [17] found the presence of HPV 16 DNA by PCR in about 30% of cases, they failed to localize HPV DNA by *in situ* hybridization in any of their PCR-based HPV positive breast carcinoma tissue sections. Also no HPV could be detected by using consensus HPV primers by Wrede *et al.* [18]. These results strongly suggest that the causal role of high risk genital HPVs, most notably the type 16 and 18, in the pathogenesis of human breast carcinoma seems to be remote. Furthermore, occurrence of HPV in cervical scrapes but not in FNAC samples of the same patient strongly indicates that though these viral genomes can putatively transform breast epithelial cells *in vitro*, they are not able to do so *in vivo*. Nevertheless, immortalization of breast epithelial cells by these HPVs will provide a good *in vitro* system for analysis of various HPV gene functions that are involved in the process of carcinogenesis.

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